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Human endothelial cell response to gram-negative lipopolysaccharide assessed with cDNA microarrays

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Zhao, Baiteng, Robert A. Bowden, Salomon A. Stavchansky, and Phillip D. Bowman. Human endothelial cell response to gram-negative lipopolysaccharide assessed with cDNA microarrays. *Am J Physiol Cell Physiol* 281: C1587–C1595, 2001.—To assess the feasibility of using cDNA microarrays to understand the response of endothelial cells to lipopolysaccharide (LPS) and to evaluate potentially beneficial agents in treatment of septic shock, human umbilical vein endothelial cells were exposed to *Escherichia coli* LPS for 1, 4, 7, 12, or 24 h. Total RNA was isolated and reverse-transcribed into ^{33}P-labeled cDNA probes that were hybridized to human GeneFilter microarrays containing ~4,000 genes. The mRNA levels of several genes known to respond to LPS changed after stimulation. In addition, a number of genes not previously implicated in the response of endothelial cells to LPS also appeared to be altered in expression. Nuclear factor-κB (NF-κB) was shown to play an important role in regulating genes identified from the microarray studies. Pretreatment of endothelial cells with a specific NF-κB translocation inhibitor eliminated most of the alterations in gene expression. Quantitative RT-PCR results independently confirmed the microarray results for monocyte chemotactic protein-1 and interleukin-8, and enzyme-linked immunosorbent assays demonstrated that augmented transcription was followed by translation and secretion.

endothelium; endotoxin; gene expression profiling

GRAM-NEGATIVE BACTEREMIA is a serious health problem and the leading cause of death in intensive care units despite the use of antimicrobial agents and advanced supportive care. About 400,000 persons are affected annually, resulting in about 100,000 deaths, making gram-negative bacteremia the 13th leading cause of death in the U.S. (1, 16). Septic shock may develop within hours after infection by gram-negative bacteria. Symptoms may include a dramatic drop in blood pressure, fever, diarrhea, and widespread blood clotting. These effects are caused in large part by circulating bacterial endotoxin (lipopolysaccharides, LPS) and the inflammatory response of the body to it. LPS is often liberated in large amounts in the circulation after bacterial lysis by antibiotics (11, 29, 33, 34). The results of LPS administration to animals and healthy volunteers provide additional support for the role of LPS in the development of septic shock (5).

LPS comprises the major component of the outer membrane of all gram-negative bacteria, and the cellular response by the host to this glycolipid appears to be an adaptive response that normally provides protection against a wide range of microorganisms by alerting cells of the immune system to their presence. The response can become magnified sufficiently to injure the host. The host can be exposed to LPS because of translocation of gut bacteria into the circulation, as a result of mucosal epithelium dysfunction or severe trauma, or through exposure by inhalation of air-borne LPS.

Endothelial cells (EC) lining blood vessels are one of the first cell types to be exposed to LPS when it is liberated into the circulation. The EC responds to LPS by producing adhesion macromolecules [E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule (VCAM)] and chemokines [interleukin (IL)-8] that direct neutrophils to the sites of inflammation. Migration of neutrophils into damaged tissue is a hallmark of the acute inflammatory response. Other as yet undiscovered mediators from host EC might also contribute to the pathogenesis of septic shock and the multiple organ dysfunction syndrome.

For LPS to be active on EC, it must form a complex with LPS binding protein (LBP) and soluble CD14 (2), both of which are present in plasma or serum. This complex is then recognized by Toll-like receptor 4 to mediate signaling transduction (4, 14, 27). CD14 can also be membrane bound (mCD14) on cell types such as monocytes and macrophages, which have been studied extensively for their roles in immune and inflammatory response. LPS-induced stimulation of EC gene expression involves the activation and nuclear translocation of several types of transcription factors, especially nuclear factor-κB (NF-κB). In nonstimulated

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cells, NF-κB is bound by its natural inhibitor IκB and stays inactive in cell cytoplasm. Upon LPS activation, IκB is degraded and NF-κB translocates into the nucleus to induce gene expression. Translocation of NF-κB can be blocked by a synthetic peptide, SN50, containing the nuclear localization sequence required for nuclear translocation of NF-κB (24). To investigate the role of NF-κB in LPS-induced cell response and to evaluate cDNA microarrays for determining drug effects, human umbilical vein EC (HUVEC) were pretreated with SN50 before LPS stimulation and were analyzed by microarrays and enzyme-linked immunosorbent assay (ELISA).

It is estimated that the human genome possesses ~26,000–40,000 genes (21, 35). Therefore, there is a high probability that other genes and their products that are not yet identified may be involved in the pathogenesis of sepsis and septic shock. This possibility is intriguing in that treatments such as antioxidants, antiendotoxin, anticytokine, ibuprofen, corticosteroids, and nitric oxide so far have not been uniformly successful in reducing the overall mortality due to gram-negative sepsis (8, 20, 26).

Recent advances in sequencing of genomes are providing high-throughput screening techniques to assess global changes in gene expression rapidly. Gene expression profiling with cDNA microarrays produced by attachment of defined DNA sequences to glass was first developed by Patrick Brown and colleagues at Stanford University (30, 31), and nylon membrane-based microarrays are now commercially available. The gene array technique provides a rapid means of identifying genes whose changing mRNA levels indicate potential alterations in expression. Continuing advances in sequencing the entire genome of organisms may ultimately make it possible to develop a comprehensive view of regulation of gene expression under various developmental, disease, or treatment states. Here we used cDNA microarrays to investigate the response of the normal human EC in vitro to LPS and to investigate means to modify this response. Nylon-based gene arrays provide an efficient technique to screen for participants in the inflammatory response and provide an important adjunct to conventional gene expression techniques for understanding gene regulation.

EXPERIMENTAL PROCEDURES

EC and treatments. First-passage cryopreserved, pooled HUVEC were obtained from Cascade Biologics (no. C-015-10C; Portland, OR), thawed, and cultured according to the supplier's recommendations in medium 200. Supplementation with the supplied growth additives resulted in a medium containing 2% fetal bovine serum (FBS), hydrocortisone, human epidermal growth factor, basic fibroblast growth factor, heparin, and penicillin-streptomycin-amphotericin B. After thawing, cells were seeded in 0.2% gelatin-coated tissue culture plate and were not used beyond *passage 5*. Cells were cultivated under 95% air-5% CO_2 at 37°C.

For the described experiment, HUVEC were seeded onto gelatin-coated six-well multiplates at 5,000 cells/cm^2 in 2.5 ml of medium per well. At confluence the medium was replaced with medium containing with 50 ng/ml of LPS (*Escherichia coli*, serotype 055:B6; Sigma, St. Louis, MO) in 10% defined FBS (Hyclone, Logan, UT) for 1, 4, 7, 12, or 24 h before harvest of total cellular RNA and supernatant. Control cells were given the same medium without LPS for 12 h. Each treatment was performed in duplicate.

For the NF-κB inhibition experiment, cells were pretreated with 50 μM NF-κB inhibitory peptide SN50 or the inactive control peptide SN50M (Calbiochem, La Jolla, CA) for 1 h before the addition of LPS at 50 ng/ml for another 4 h. The parallel controls were given the same medium without any peptide for 1 h, and then LPS was added for the positive control or not added for the negative control and the controls were incubated for another 4 h. At the end of treatments, total cellular RNA was isolated and cell culture supernatants were collected. Each treatment was done in a single well.

Total RNA isolation. Total cellular RNA was isolated from cultured HUVEC using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. The RNA yield was determined spectrophotometrically with a SpectraMAX 250 (Molecular Devices, Sunnyvale, CA). The quality of the RNA was determined based on absorbance at 260 and 280 nm and gel electrophoresis on 0.9% agarose containing 1:10,000 SYBR Gold nucleic acid stain (Molecular Probes, Portland, OR). Only undegraded RNA free of genomic DNA contamination was used. Isolated RNA was heated to 70°C for 5 min, quickly frozen on liquid nitrogen, and lyophilized until ready for ^{33}P labeling and RT-PCR.

cDNA production for interrogation of microarray for alteration in gene expression. Two micrograms of lyophilized total RNA were suspended in 8 μl of H_2O and 2 μl of oligo(dT) (1 μg/μl of 10- to 20-mer mixture, Research Genetics), heated at 70°C for 10 min, and then briefly chilled on ice. A master mix containing 6 μl of 5× first-strand buffer, 3 μl of 0.1 M dithiothreitol, 1.5 μl of reverse transcriptase (200 units/μl, SuperScript II RT; Life Technologies, Gaithersburg, MD), 0.6 μl of dNTP mixture (dCTP, dTTP, and dGTP at 15 mM each), 1 μl of RNase inhibitor (Prime RNase Inhibitor 30 units/μl; Eppendorf, Westbury, NY), and 1 μl of [^{33}P]dATP (10 mCi/mmol; Amersham-Phamacia, Arlington Heights, IL) was added to the primed RNA in a final volume of 30 μl. The reaction was carried out at 39°C. At the end of 90 min, the reaction was stopped by addition of 4 μl of 0.5 M EDTA, 4 μl of 1 M NaOH were added, and the sample was heated to 68°C for 20 min to hydrolyze the RNA. Ten microliters of 1 M Tris·HCl (pH 7.4) were added to neutralize the sample, and the labeled probe was separated from unbound nucleotides by passage through a centri-spin-20 column (Princeton Separations, Adelphi, NJ) at 750 *g* for 2 min.

cDNA microarray hybridization and image acquisition. The GF211 GeneFilter cDNA microarray used in the described experiments contains ~4,000 known human genes on a 5 cm × 7 cm nylon filter with pore size of 0.45 μm. Each spot on the membrane contains ~0.5 ng of insert DNA from an IMAGE/LLNL cDNA clone containing the 3′ end of a gene. The insert cDNA has been denatured and ultraviolet cross-linked to the positively charged membrane. Hybridization was performed according to the manufacturer's recommendation. Blocking of nonspecific binding to the membranes was accomplished with 5 ml of MicroHyb hybridization solution containing 1.0 μg/ml poly(dA) (Research Genetics) and 1.0 μg/ml human Cot-1 DNA (BRL-Life Technologies) in a tube rotisserie at 42°C for 2 h before the labeled cDNA was added. At the end of the ~20- to 24-h hybridization period, two washes in 30 ml of 2× SSC (1× SSC = 150 mM NaCl and 15 mM trisodium citrate), 1% SDS at 50°C for 20 min were performed, followed by one wash in 100 ml of 0.5× SSC, 1%

SDS for 15 min at room temperature. The membranes were then placed on Tris-EDTA (10 mM-1 mM) buffer-moistened Whatman filter paper and wrapped with plastic wrap before exposure to a phosphoimager screen (Packard Instruments, Meridian, CT). After appropriate exposure, a high-resolution image (43 μm) was obtained by scanning in the Cyclone phosphoimager, and images were acquired with the manufacturer's software, Optiquant (Packard Instruments).

Data analysis. The relative intensities of the clones on the acquired images of the microarrays were analyzed with Pathways 3.0 software (Research Genetics) and compared with the nontreated control. Differences in the quantification of RNA and cDNA labeling were taken into account by normalization between arrays by dividing the intensity of each clone on one array by the mean intensity of all clones on that array except the control points (total genomic DNA spots).

A statistical analysis, the Chen test (7) provided by the software, was used to determine whether the expression ratio (LPS-exposed/nonexposed cells) from two microarrays or from two conditions (each represented by duplicate microarrays) deviated outside the 99.9% confidence interval for chance-observed magnitudes of the expression ratio. The limits of this interval were taken as the screening threshold. To identify these limits in terms of expression ratio, this test (7) was applied to the duplicate mean expression intensity for each gene in control conditions with respect to the duplicate mean of the same gene after a given time of LPS exposure of the cells. The assumption of this statistical test is that the coefficient of variation is constant across all microarray data points. To avoid false positives from misalignments and irregular hybridization patterns, each individual clone whose expression changed significantly was visually checked on the computer screen.

Real-time quantitative RT-PCR. One microgram of total RNA from the same sample used for microarray was reverse-transcribed to cDNA with Superscript II reverse transcriptase and poly dT priming according to the manufacturer's instruction (Life Technologies). Real-time quantitative PCRs were performed with a LightCycler thermal cycler (Idaho Technology, Salt Lake City, UT) in a total reaction volume of 8 μl containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.0–3.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM sense and antisense primers, 1 μl of 1:1,000 SYBR Green I (Molecular Probes), 1 μl of cDNA solution from reverse transcription, and 0.45 U of Klen*Taq* DNA polymerase (AB Peptides, St. Louis, MO). The gene-specific primers and sizes of PCR products were glyceraldehyde 3-phosphate dehydrogenase sense TCCTGCACCACCAACTGCTTAG, antisense TGCTTCACCACCTTCTTGATGTC, 341 bp; monocyte chemotactic protein-1 (MCP-1) sense CCTCCAGCATGAAAGTCTCTGC, antisense AGTGTTCAAGTCTTCGGAGTTTGG, 313 bp; IL-8 sense ATGACTTCCAAGCTGGCCGTGGCT, antisense TCTCAGCCCTCTTCAAAAACTTCTC, 289 bp. The conditions of the PCR reactions varied for different primer sets and were empirically determined for each primer pair. For all PCR amplification, the sample was preheated at 94°C for 15 s and received 30–45 cycles of 0 s at 94°C, 0 s at 58–60°C, 12–15 s at 72°C and then 1 min at 74°C for a final elongation; the melting curve was generated by gradually increasing temperature from 74°C to 96°C at 0.1°C/s. Fluorescence from reaction cuvettes was read at the end of each cycle and continuously during the melting curve step. Quantification of cDNA samples was achieved by reference to a standard curve generated from a series of dilutions of positive control DNA with known copy number. Positive control DNA for each gene was generated by two rounds of PCR and gel purification and quantified by reference to quantitative DNA marker (BRL-Life Technologies) on 2% agarose gel.

ELISA. Cell culture supernatants were collected at the end of treatment and stored at −20°C until being analyzed by ELISA for monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8) (R&D Systems, Minneapolis, MN). Samples were appropriately diluted before assay according to the manufacturer's instructions. The detection sensitivity is 5 pg/ml for MCP-1 and 10 pg/ml for IL-8.

RESULTS

Stimulation of HUVEC by LPS. Early experiments with LPS treatment indicated that it induced large increases in mRNA for MCP-1 and IL-8, and these two cytokines were used to define optimal conditions for cell treatment. To obtain reproducible results with the HUVEC required careful attention to the cultivation of the cells. Certain batches of media and FBS caused high background in MCP-1 and IL-8 as measured by ELISA in untreated cells. The medium and supplementation provided by Cascade Biologics consistently produced low background and yielded reproducible results. With the use of EC derived from at least three donor umbilical cords, interindividual variation in LPS responsiveness was reduced. Although hydrocortisone is a component of this medium, removal for 48 h before the experiment did not change the results (data not shown), indicating that hydrocortisone at 1 μg/ml did not act as an anti-inflammatory agent in this cell type. The 50 ng/ml concentration of LPS was chosen for this study because preliminary experiments showed that ~90% of maximum induction of MCP-1 secretion was achieved at this dose. The LPS-induced HUVEC response was sensitive to serum concentration as it provides both LBP and soluble CD14, and 10% FBS was used during LPS stimulation (Fig. 1).

Assessment of reproducibility of microarrays. Preliminary experiments with high-density microarrays indicated that reproducible results in terms of relative

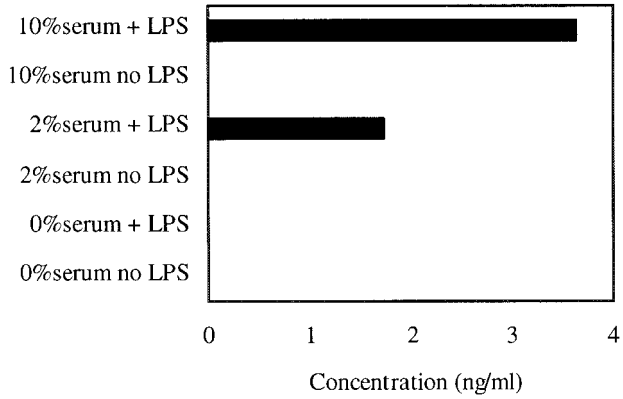

Fig. 1. Serum effect on lipopolysaccharide (LPS)-induced monocyte chemotactic protein-1 (MCP-1) secretion in human umbilical vein endothelial cells (HUVEC). Confluent HUVEC in 6-well multiplates were treated with or without 50 ng/ml *Escherichia coli* LPS in medium with various amounts of fetal bovine serum (0%, 2%, and 10%) for 4 h. Cell culture supernatants were collected and analyzed by MCP-1 enzyme-linked immunosorbent assay (ELISA). LPS-induced MCP-1 secretion is highly dependent on serum concentration.

changes in gene expression required labeling of samples to be done at the same time with the same master mix and required hybridization to be done on the same lot of membranes and exposed for the same period of time. To assess the reproducibility of results obtained with microarrays, total cellular RNA from duplicate samples treated without (control) and with LPS for 1, 4, 7, 12, or 24 h was isolated individually and separately radiolabeled by poly dT-primed reverse transcription and then hybridized to microarrays.

Comparison of normalized intensities between control duplicates is shown in Fig. 2*A*. The two parallel lines define a 99.9% confidence interval for random variation of the clone on the ordinate axis. Only three clones, representing approximately the expected 0.1% of total clones, were outside the confidence limits. The close fit between duplicates (all $r^2 > 0.99$) suggested that independent handling of different samples, i.e., RNA isolation, cDNA labeling, and hybridization to different membranes, introduced very little variation. Figure 2*B* illustrates the comparison between controls and 4-h LPS-treated samples using the mean intensity of duplicates. In this case, 19 clones (representing 17 different genes) produced an expression ratio (to untreated controls) that was beyond the screening threshold set at the 99.9% confidence limits for random variation. The use of mean intensities for each gene from duplicate microarrays reduces the distribution variation and results in a narrower confidence interval. Duplicate microarray results were used for all the expression ratio results in this study. The screening threshold represented in all comparisons an ~1.6-fold up- or downregulation.

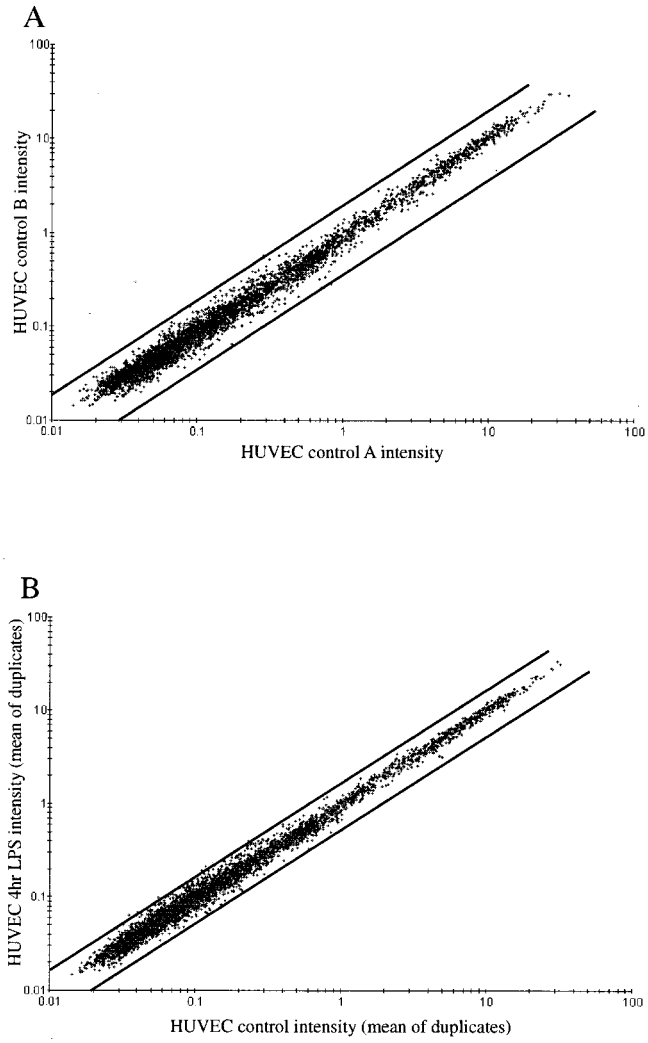

Fig. 2. Correlations of microarray analysis between hybridizations for 2 separate control duplicates (*A*) and for duplicate means of controls and samples treated with LPS for 4 h (*B*). Total cellular RNA was isolated, reverse-transcribed, and hybridized to 2 microarrays. The intensities of acquired images were analyzed by computer program. A spot outside of the 2 parallel lines represents a gene outside the 99.9% confidence limit (screening threshold).

Gene expression profiling of LPS-modulated genes. By comparing LPS-treated samples to control samples over time, we found the expression ratios (after stimulation) for a small number of genes to be beyond the screening threshold limit. Their relative fold changes ranged from 0.3 to 0.4 for downregulation and from 1.6 to 11.3 for upregulation at different times as shown in Table 1. These 38 genes account for ~1% of all the genes profiled and were arranged based on initial time when showing significant changes, with reverse-highlighted ratios indicating fold changes beyond the screening threshold at the times indicated. The variation between the 38 genes in duplicate experiment was assessed by calculating the percent difference between duplicate intensities of each gene and their mean at each treatment time point. The mean intensity difference between duplicates was 7% with a 6% standard deviation. The correlation coefficient for the duplicate 38 genes at all time points was 0.97. Among the LPS-affected genes, some of them are known for their roles in inflammatory responses. For example, IL-8, MCP-1, and fractalkine are CXC, CC, and CX3C type chemokines, respectively. VCAM-1 is an adhesion molecule. Plasminogen activator inhibitor (PAI) types I and II are procoagulation factors. The other genes identified are less well studied or not previously studied for their roles in the EC response to LPS. On the basis of current information, these other genes identified by alteration beyond the microarray screening threshold belong to groups including chemoattractants (e.g., IL-8, MCP-1, SCYD1, LOXL2), extracellular matrix-related proteins (e.g., PAI-1, PAI-2, MMP10, LAMC2), inflammation (e.g., NK4, HLA-C, B2M, TAPBP)-, signal transduction (e.g., PRKAR2B, MARCKS, CAV1)-, transcription and translation (e.g., ZFP36, STAF50, PC4, HNRPA2B1, SNRPG, EIF4A1)-, protein trafficking (e.g., ARHB, ARF4, SEC61B)-, and metabolism (e.g., LDHA, SAT, EXT1, NNMT, NP)-related genes, and genes without clearly known function (e.g., NMA). LPS-induced changes of gene expression appeared to be time dependent.

To validate particular microarray screening results and to assess the correlation between hybridization and RT-PCR methods, real-time quantitative PCR was performed for MCP-1 and IL-8. One microgram of total RNA from the same preparation for microarray analy-

Table 1. *Gene expression profiling of LPS-stimulated HUVEC with microarrays*

1 h	4 h	7 h	12 h	24 h	Acc	Title	Abbr.	SN50
0.3	0.3	0.4	0.6	0.8	AA180007	Protein kinase, cAMP-dependent, regulatory type II β	PRKAR2B	1.6
0.4	0.7	0.7	0.7	0.7	R56149	Putative transmembrane protein	NMA	
1.8	1.5	1.3	1.1	0.9	H05914	Lactate dehydrogenase A	LDHA	
1.8	0.9	0.9	0.9	0.8	R38383	Zinc finger protein homologous to Zfp-36 in mouse	ZFP36	
2.1	2.3	1.8	1.1	0.9	H11003	Endothelin 1	EDN1	1.1
1.8	5.4	3.3	1.4	1.1	AA102526	Interleukin 8	IL-8	1.0
2.5	2.7	1.8	1.6	1.1	AA495790	ras homolog gene family, member B	ARHB	1.2
3.0	11.3	8.3	3.9	2.0	AA425102	Monocyte chemotactic protein-1	MCP-1	1.5
1.0	1.8	1.2	1.0	1.1	R66139	Small inducible cytokine subfamily D (Cys-X3-Cys), member 1 (fractalkine, neurotactin)	SCYD1	1.0
1.1	1.7	1.7	1.4	1.1	N54794	Plasminogen activator inhibitor, type I	PAI-1	1.1
0.9	2.0	1.1	0.7	0.8	H16591	Vascular cell adhesion molecule 1	VCAM-1	1.0
1.1	2.1	1.4	1.0	1.1	AA011215	Spermidine/spermine N1-acetyltransferase	SAT	0.8
1.0	1.7	1.7	1.3	1.0	AA482231	Myristoylated alanine-rich protein kinase C substrate	MARCKS	1.0
1.7	2.2	2.2	1.2	1.0	AA055835	Caveolin 1, caveolae protein, 22 kDa	CAV1	1.0
1.1	2.2	2.2	1.2	1.0	AA487582	Exostoses (multiple) 1	EXT1	0.9
0.8	1.8	1.7	1.0	0.9	T72089	Nicotinamide *N*-methyltransferase	NNMT	1.3
1.0	1.8	2.5	2.6	1.4	AA458965	Natural killer cell transcript 4	NK4	1.4
0.9	1.9	2.6	2.1	1.6	AA464246	Major histocompatibility complex, class I, C	HLA-C	1.3
1.1	1.6	2.2	2.0	2.1	AA670408	β-2-Microglobulin	B2M	1.0
1.4	1.6	1.8	1.4	1.9	AA083478	Stimulated *trans*-acting factor (50 kDa)	STAF50	1.0
0.9	1.1	1.8	1.0	0.9	AA099534	Activated RNA polymerase II transcription cofactor 4	PC4	
0.8	1.2	1.7	0.9	0.9	W02101	Heterogeneous nuclear ribonucleoprotein A2/B1	HNRPA2B1	
1.0	1.3	1.7	1.3	1.3	AA133577	Small nuclear ribonucleoprotein polypeptide G	SNRPG	
1.2	1.4	1.7	1.2	1.1	H09590	Eukaryotic translation initiation factor 4A, isoform 1	EIF4A1	
1.0	1.3	1.7	1.2	1.1	H07880	Chaperonin containing TCP1, subunit 6A (ζ1)	CCT6A	
0.9	1.1	1.8	1.2	1.2	T71316	ADP-ribosylation factor 4	ARF4	
1.0	1.0	1.8	1.6	1.0	H73928	Protein translocation complex-β	SEC61B	
1.0	1.3	1.9	1.2	1.1	T65118	Catenin (cadherin-associated protein), α1 (102 kDa)	CTNNA1	
0.8	1.5	1.7	1.3	1.0	AA410604	CDC16 (cell division cycle 16, *Saccharomyces cerevisiae,* homolog)	CDC16	
0.9	1.5	1.9	1.0	0.8	T49159	Plasminogen activator inhibitor, type II (arginine-serpin)	PAI-2	
0.9	1.6	2.1	1.4	1.2	AA857496	Matrix metalloproteinase 10 (stromelysin 2)	MMP10	
1.0	1.2	2.0	1.1	1.1	N80129	Metallothionein 1L	MT1L	4.8
1.0	1.0	1.7	1.2	1.1	AA430382	Nucleoside phosphorylase	NP	
1.1	1.4	1.8	1.3	1.0	AA676458	Lysyl oxidase-like 2	LOXL2	
1.2	1.5	2.1	1.6	1.2	AA047338	Proteasome subunit, α-type, 6	PSMA6	
1.2	1.6	2.5	2.3	2.6	AA677534	Laminin, γ2 nicein (100 kDa), kalinin (105 kDa)	LAMC2	
0.8	0.8	1.2	1.6	1.3	AA463257	Integrin, α2	ITGA2	
0.9	1.1	1.4	1.6	1.1	T69304	TAP binding protein (tapasin)	TAPBP	

Confluent human umbilical vein endothelial cells (HUVEC) were treated with *Escherichia coli* lipopolysaccharides (LPS) for various times or left untreated as controls. Total cellular RNA was isolated, reverse-transcribed into ^{33}P-labeled cDNA, and hybridized to GF211 GeneFilter microarrays containing ~4,000 genes. The data are expression ratios (post-LPS/no LPS). At various times, 38 genes (highlighted) were determined to be outside the screening threshold of 99.9% confidence limits for random variation. The expression ratios of corresponding genes from the nuclear factor-κB (NF-κB) inhibition experiment are listed in the last column (SN50 is the inhibitor used). Most of the genes affected by 4-h LPS treatment appeared to be NF-κB dependent. Acc, GenBank accession no; Abbr., abbreviation.

sis was reverse-transcribed and amplified on a LightCycler as described in *Real-time quantitative RT-PCR*. Although the fold changes shown by RT-PCR were considerably greater than those from microarrays (Table 2), the patterns are similar between the two techniques.

To determine whether changes in gene transcription resulted in changes in gene translation, the secretion of MCP-1 and IL-8 into culture supernatant was evaluated by ELISA (Fig. 3). The basal levels of cytokine secretion from controls were minimal compared with those from LPS-treated samples.

Effect of NF-κB inhibition on gene expression. Confluent EC were pretreated with 50 μM synthetic inhibitory peptide (SN50) or control peptide (SN50M) for 1 h before addition of 50 ng/ml LPS and were incubated for another 4 h. A negative control (no peptide, no LPS) and an LPS-treated positive control (no peptide) were done in parallel. At the end of the 4-h incubation, the

Table 2. *Comparisons between results from RT-PCR and microarray analysis*

	MCP-1		IL-8	
	RT-PCR	Microarray	RT-PCR	Microarray
1 h LPS/CTL	215	3.0	40	1.8
4 h LPS/CTL	948	11.3	104	5.4
7 h LPS/CTL	427	8.3	53	3.3
12 h LPS/CTL	164	3.9	23	1.4
24 h LPS/CTL	31	2.0	6.7	1.1

The mRNA expression of monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8) was analyzed with both real-time quantitative RT-PCR and microarrays. The expression ratios by RT-PCR were generated by comparing copy number of MCP-1 or IL-8 mRNA to controls after normalizing to housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The expression ratios by microarray analysis were determined by computer program based on the normalized intensities. The denominator (CTL) of all expression ratios is defined by the result from control cells not exposed to LPS.

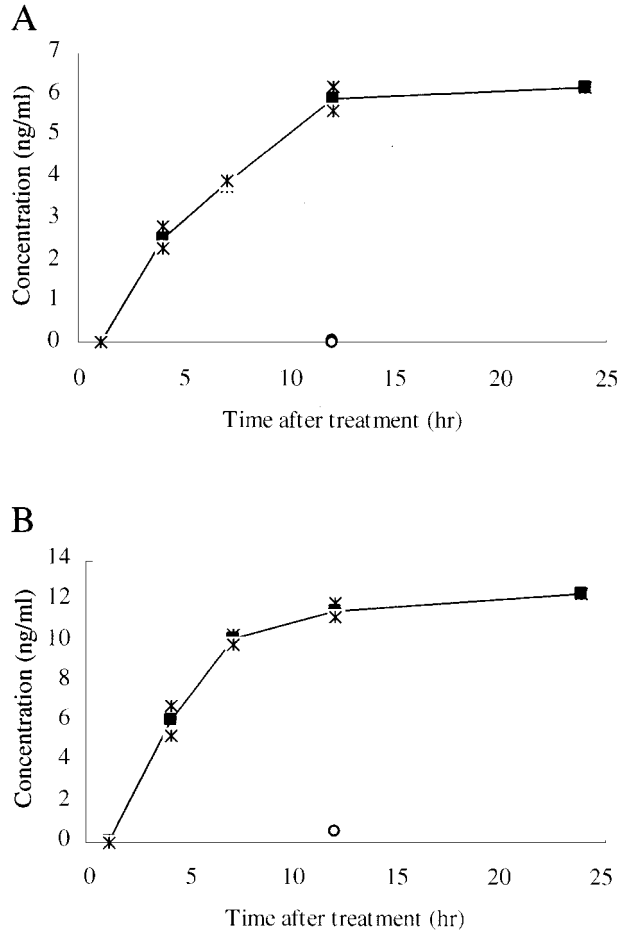

Fig. 3. LPS-induced MCP-1 and interleukin (IL)-8 secretion. Confluent HUVEC in 6-well multiplates were treated with 50 ng/ml *E. coli* LPS (■) for various times. Negative controls (○) were incubated for 12 h. Cell culture supernatants were collected and analyzed by MCP-1 and IL-8 ELISA. Results are shown from duplicate samples at each time point (*), and the means were connected.

supernatants were collected and RNA was isolated for microarray analysis as described above. SN50 is able to block the nuclear translocation of NF-κB complex, thereby preventing it from activating gene expression. That 1-h pretreatment was sufficient to abolish almost all NF-κB activation is shown in Fig. 4 based on the mRNA expression ratios (by microarray analysis) and secretion of MCP-1 and IL-8. Pretreatment with SN50 inhibited all genes whose expression was affected at 4 h by LPS except MT1L. Pretreatment with SN50M did not affect the stimulation of gene expression by LPS. SN50M treatment alone caused some changes in several genes: metallothionein 1E (MT1E) appeared stimulated; eukaryotic translation elongation factor 1-γ (EEF1G) and centractin-α (ACTR1A) appeared depressed. The great majority of the genes on the microarray, however, were not affected by NF-κB inhibition.

DISCUSSION

The EC is one of the first cell types to contact and respond to circulating LPS released from gram-negative bacteria. The expression levels of a variety of genes have been found to change in LPS-stimulated EC. The cellular response to LPS appears to be an adaptive response of the host that normally provides protection against a wide range of microorganisms, but if too vigorous it may cause injury. LPS-stimulated cytokines such as tumor necrosis factor (TNF)-α, IL-1, and IL-6 have been implicated in mediating this inflammatory state that results in the pathogenesis of septic shock and the multiple organ dysfunction syndrome in which EC actively participates. Clinical trials attempting to negate the activity of the known mediators implicated in septic shock have not proven successful (8, 20, 26). The current failure in reducing overall mortality may be due to an incomplete understanding of the pathophysiology involved. There are probably other gene products involved in the cellular responses to endotoxin with as yet unknown roles.

The recently developed gene microarray technology provides a rapid screening procedure to explore parallel alterations of gene expression through comparative analysis of mRNA expression of a large number of genes. Nylon membrane-based GeneFilter microarrays containing ~4,000 known human genes were used in this study. To obtain consistent results, RNA quality and cDNA labeling must be closely monitored and all samples must be processed at the same times and conditions with microarrays from the same lot. Despite the presence of expression data for a large number of genes, and the use of all of them to determine a common random confidence interval applied to each gene in a comparison set (7), there is a lack of gene replicate variation beyond that contained in duplicate microarrays. Thus the chances are not assessed that certain genes exhibiting large observed deviation from control value in one run might not be those exhibiting such deviation if other microarray runs were possible at a given time point. Consequently, the statistical approach does not allow meaningful determination of significance for a given gene's response, but it does allow arbitrary assignment of a threshold deviation beyond which a chance deviation is unlikely. Thus, as used here, microarrays provide only a screening technique to identify genes for future testing. We have used a screening threshold at the 99.9% confidence limits for random variation in an arbitrary compensation for evaluation of the large multiplicity of genes, each without iteration beyond duplicates for a given time point.

On the basis of the quantitative RT-PCR results, there were ~1×10^5 copies of MCP-1 transcripts per 1 μg of total cellular RNA in control samples, which translates into ~2–3 copies of messages per cell. When 2 μg of total RNA was used in microarray analysis, that expression level was readily detectable on one membrane by the conservative criterion of a visually perceptible spot discriminated clearly from background. RT-PCR results exhibited greater fold changes than the microarray analysis for the two genes tested. This discrepancy may come from the different sensitivities and detection limits of the two techniques.

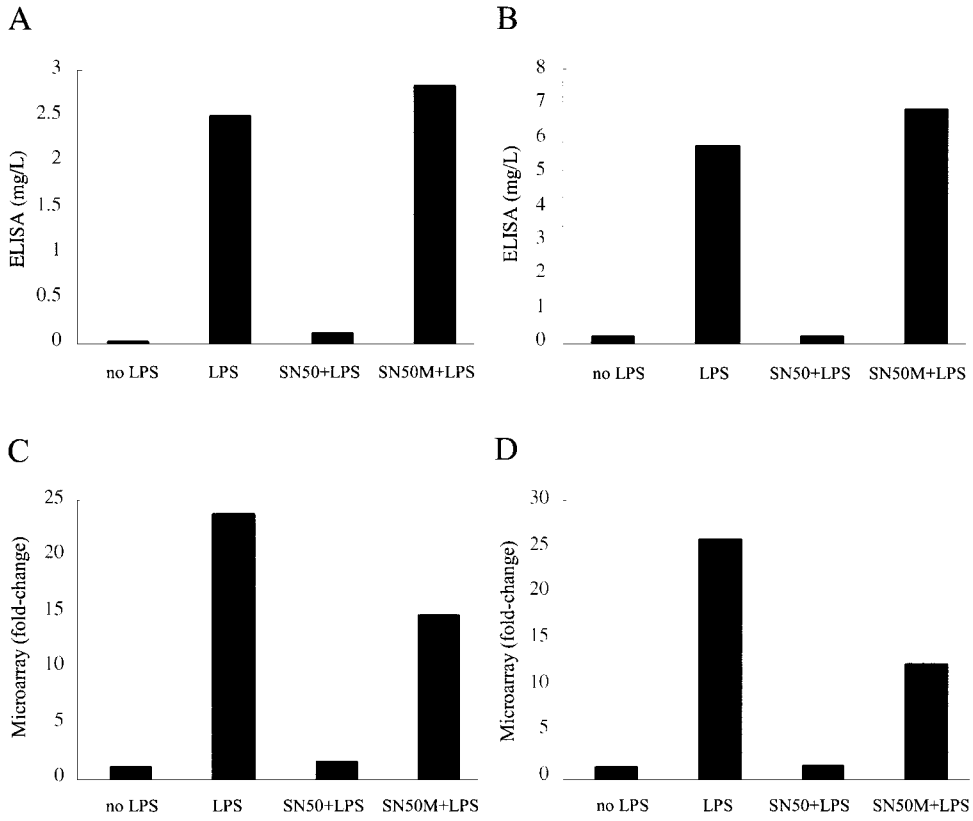

Fig. 4. Effects of nuclear factor-κB (NF-κB) inhibition on cytokine secretion. Confluent HUVEC in 6-well multiplates were pretreated with 50 μM inactive control peptide SN50M (SN50M + LPS) or 50 μM inhibitory peptide SN50 (SN50 + LPS) for 1 h before being given 50 ng/ml LPS for 4 h. A negative control (no LPS) and a positive control (LPS) were performed in parallel. Cell culture supernatants were collected at the end of treatments and analyzed by ELISA and for MCP-1 (*A*) and IL-8 (*B*). Their mRNA expression fold changes with respect to negative control (no LPS) are shown from microarray analysis for the same samples [MCP-1 (*C*) and IL-8 (*D*)].

Screening in the present study was limited to ~4,000 known genes or 10–15% of the estimated number of genes in the human genome (21, 35). Genome-wide expression screening cannot be done until all genes are identified and placed on microarrays or DNA chips. By comparing mRNA levels after exposure of HUVEC to LPS, 38 of the 4,000 genes on the cDNA microarray were screened as positive for change due to LPS. The identification of several genes previously described as involved in inflammation supports the ability of microarray analysis to identify new genes associated with inflammation. Extrapolation to the entire genome indicates that up to several hundred genes might be affected by LPS stimulation of EC.

In addition to some of the well-known LPS-induced genes, microarray analysis also suggested the involvement of a number of genes whose roles were poorly understood or not understood in EC during inflammatory response. For example, the upregulated lysyl oxidase-like 2 (LOXL2) gene is a member of the lysyl oxidases (LO), which modify elastin and collagen by catalyzing the oxidization of some lysine residuals within proteins and initializing subsequent cross-linking (37). Evidence also showed that LO could have certain biological roles in cell nuclei (23), and purified LO from calf aorta was a potent chemoattractant to human monocytes (22). Thus LOXL2, structurally and functionally similar to LO, may also possess chemotactic ability and other unknown functions in inflammation.

Spermidine/spermine N1-acetyltransferase (SAT), whose function is to regulate polyamine metabolic pathway (6), also appeared to be induced by LPS. Its substrates, polyamines, are thought to have several vital roles in cellular regulation. Spermine inhibits proinflammatory synthesis of cytokines such as TNF, IL-1, IL-6, macrophage inflammatory protein (MIP)-1α, and MIP-1β (39) and LPS-induced nitric oxide release (32). The mechanism of counterregulating cellular activation in inflammatory response indicates a need to maintain intracellular spermine concentration (38), but the increased SAT may adversely reduce the amount of spermine and thus promote the cellular activation. An inhibition of this enzyme may help the cell retain and counterregulate its inflammatory response.

Exostoses (multiple) 1 (EXT1), involved in the biosynthesis of heparan sulfate glycosaminoglycans (GAG) may be important in regulating vascular permeability, thromboresistance, and cellular interactions. IL-1 and TNF caused an increase of GAG in cell culture supernatant and a reduction in cell-associated GAG (19). An animal study showed a marked increase in heparan sulfate and GAG synthesis during an acute inflammatory reaction to LPS (25). Therefore, GAG could play an important role in LPS-induced cellular response. The altered gene expression of EXT1 may implicate the cause of all these changes in GAG biosynthesis.

LPS-stimulated EC appeared to stimulate genes for a group of major histocompatibility complex (MHC) molecules, including HLA-C, B2M, and TAPBP. HLA-C and B2M are the components of class I MHC that involves the presentation of foreign antigens to

the immune system (3). TAPBP helps assemble of MHC-TAP complexes. TAP, an ATP-binding transporter, facilitates the transportation of proteasome complex processed peptides to MHC molecules (17). A subunit of the proteasome, PSMA6, was as well identified as upregulated. Thus EC activated the antigen-presenting pathway and may be subject to the cytotoxicity of $CD8^+$ T cells. To facilitate leukocyte extransvasation from blood to tissue in inflammatory response, EC actively adjusted its gene expression. LPS appeared to activate a member of the matrix metalloproteinases (MMP), MMP10 in HUVEC. MMP are a family of proteases against components of the extracellular matrix (ECM). Their main function is the degradation of ECM, thus modulating cell adhesion and migration. One MMP is able to stimulate chemoattraction through the cleavage of laminin 5γ2 chain (15), which was also upregulated in EC by LPS stimulation. Integrins are important in regulating the expression of some MMP expression. An integrin member, α2β1, is capable of activating a stress- and cytokine-related mitogen-activated protein kinase (MAPK), p38, and the induction of MMP13 (28). The α2 subunit (ITGA2) of this integrin showed increased expression at mRNA level after LPS stimulation. The expression of another MMP member, MMP2, can also be induced by LPS in EC (18). Together, our results demonstrate that EC responded to LPS by regulating their gene expression in such a way that a network of many pathways was activated, ostensibly cooperating to counteract the insult of bacterial invasion. Microarray analysis may be an effective screening tool for identifying these cellular events.

Several studies have applied gene expression analysis with cDNA arrays to the responses of cells to whole bacteria. Cohen et al. (9) reported the response of the human promyelocytic cell line THP1 to the intracellular gram-positive bacteria *Listeria monocytogenes* using three different arrays and found ~100 genes to be up- or downregulated. Eckmann et al. (12) used the same type of cDNA microarray we used to study the responses of two human epithelial cell lines to the gram-negative bacteria *Salmonella*. After 3, 8, or 20 h of bacterial coculture, 50 genes were found to be stimulated. Among them, two genes (IL-8 and HLA-C) were also found to be upregulated in our study. Coombes and Mahony (10) found that ~8% of 268 genes studied were upregulated when *Chlamydia pneumoniae* (an obligate intracellular gram-negative bacterium) was added to human microvascular EC. Similar to our findings, IL-8 and MCP-1 were among the most prominently stimulated genes in EC responding to this bacterium. There were very few genes altered in their expression that were in common among the responses of these different cell lines to different bacteria. This suggests that cells of different tissues respond differently to whole bacteria or bacterial cell wall products or that different bacteria or their products may differentially effect expression of mammalian cells.

It has been shown that the mechanisms of LPS-induced alterations in gene expression involve several transcription factors. NF-κB appears to be important for inducing many inflammation-related genes, such as MCP-1 and IL-8 (13, 36). Here we investigated the role of NF-κB on gene expression in LPS-stimulated EC using microarray analysis. Strikingly, the inhibition of NF-κB appeared to abolish nearly all of the LPS-induced alterations of gene expression in EC at 4 h. Some genes are under the direct control of NF-κB, whereas others may be indirectly regulated. The importance of NF-κB in regulating the expression of many inflammation-related genes was also confirmed in the response of epithelial cells to bacterial infection (12).

In conclusion, LPS appeared to alter the expression of a number of genes in human EC. In addition to a number of genes already identified, microarray analysis demonstrated the ability to identify more genes. We confirmed that NF-κB is an important transcription factor involved in the inflammatory reaction in EC and that the effect of an agent (SN50) can be monitored using cDNA microarray analysis. Although the ability of nylon-based microarrays to detect fold changes may not be as great as that of RT-PCR, their ability to globally identify candidate genes for involvement in cellular perturbations makes them an important new adjunct in understanding processes involving transcription activation.

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